Chapter 15

Gene Expression Studies on Human Trisomy 21 iPSCs and Neurons: Towards Mechanisms Underlying Down's Syndrome and Early Alzheimer's Disease-Like Pathologies

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Abstract

The cause of Alzheimer disease (AD) is not well understood and there is no cure. Our ability to understand the early events in the course of AD is severely limited by the difficulty of identifying individuals who are in the early, preclinical stage of this disease. Most individuals with Down's syndrome (DS, trisomy 21) will predictably develop AD and that they will do so at a young age makes them an ideal population in which to study the early stages of AD. Several recent studies have exploited induced pluripotent stem cells (iPSCs) generated from individuals with familial AD, spontaneous AD and DS to attempt to identify early events and discover novel biomarkers of disease progression in AD. Here, we summarize the progress and limitations of these iPSC studies with a focus on iPSC-derived neurons. Further, we outline the methodology and results for comparing gene expression between AD and DS iPSC-derived neurons. We highlight differences and commonalities in these data that may implicate underlying genes and pathways that are causative for AD.

Key words Pluripotent stem cells, Microarray, Expression analysis, Neurons, Down's syndrome, Alzheimer's disease

1 Introduction

Alzheimer's disease (AD) is characterized by progressive dementia associated with amyloid plaque formation, neurofibrillary tangles (NFTs) and cortical neuron degeneration. AD typically begins with subtle memory failure that becomes more severe and is eventually incapacitating. Amyloid plaques and NFTs are considered late events in AD pathology, so defining the initial events in AD pathology is key to understanding its progression. A number of hypotheses have been proposed as to the earliest changes that underlie AD symptoms. For instance, synapse loss is believed to be one of the earliest events in neurodegeneration associated with AD [1] and has been substantiated by the decreased number of

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synapses in post-mortem AD brain [2–5]. Oxidative stress (OS) has also emerged as a potential early systemic trigger of AD pathology, where post-mortem AD brains and those of AD animal models show indications of oxidative damage [6–11].

Defining the root cause of AD, whether it is triggered by oxidative stress, synapse loss, Aβ deposits, tau phosphorylation or other mechanisms relies on the ability to analyze the earliest events in AD. Yet, the study of disease progression in AD has been hindered by the fact that diagnosis is confirmed by post-mortem evidence of amyloid plaques in the brain [12, 13]. Furthermore, most individuals who are diagnosed are no longer in early stages of the disease. Because the age of onset of AD is generally over age 65, the first symptoms are often mistakenly attributed to aging or stress. This makes it difficult to identify individuals who are in early stages of AD and to define early events in the disease. Individuals with many familial forms usually develop AD symptoms between 50 and 65 years of age, providing an early window into AD. Yet, early-onset familial AD is relatively uncommon, accounting for about 5 % of total Alzheimer's disease [14, 15] or about 250,000 cases in the United States.

In addition to familial AD caused by single gene mutations or duplications, individuals with Down's syndrome (DS) develop AD before age 65 and provide a good model for studying AD pathology progression. The incidence of DS in the US is approximately 1 in 1,200 births resulting in a total of approximately 250,700 individuals with DS in the U.S. [16]. Adults with DS are at an extremely high risk for developing AD, with most individuals over age 40 showing amyloid deposits (based upon autopsy findings) and over half of DS adults older than 60 years of age diagnosed with AD [17–26].

DS is caused by triplication of human chromosome 21 (Hsa21) and many AD candidate genes are located on Hsa21. These genes include *APP*, *DYRK1A*, and *SOD1*. It is believed that the presence of an extra copy of APP on chromosome 21 provides more substrate for production of Aβ peptide and puts individuals with DS at considerably greater risk than the general population for early Aβ plaque deposition and the appearance of AD symptoms. In support of this notion, mice that overexpress only human APP develop early biochemical and cognitive hallmarks of AD [27]. Dual specificity tyrosine-phosphorylation-regulated kinase 1A (*DYRK1A*) can phosphorylate Tau [28] and may therefore be involved in its hyperphosphorylation and subsequent aggregation. Superoxide dismutase 1 (*SOD1*) is responsible for destroying free superoxide radicals and its imbalance may affect levels of oxidative stress. In addition, with up to 500 genes located on Hsa21, it is possible that other genes contribute to the progression of AD in DS individuals. Elucidation of the mechanisms of these genes can inform both disease progression and potential therapeutic strategies.

Taken together, there are several advantages to studying AD progression in individuals with DS: (1) DS is diagnosed at birth (or prenatally), (2) DS is a predictor of individuals who will likely develop AD, (3) DS affects more people than early onset familial AD, (4) DS individuals develop AD symptoms before age 40, and (5) many AD candidate genes are encoded by chromosome 21. These traits make DS individuals a unique population in which to examine early stages of AD progression and identify early biomarkers.

As described, it is crucial to define the earliest events in AD so as to study disease cause and progression. DS individuals provide a unique population that will reliably develop AD at an early age that can be used to study early neuropsychological and biochemical events in AD. Yet, there remains the problem of developing a system in which to identify early cellular and molecular events in AD pathology. Induced pluripotent stem cell (iPSC) technology allows the creation of disorder-specific human cells to define errors in human neurodevelopmental and neurodegenerative disease [29–31]. The application of iPSCs to AD has been demonstrated by recent studies that identified cellular pathologies in AD neurons as well as altered gene expression patterns [32–36] (see below). In addition, iPSCs and their neuronal derivatives have been used to identify early cellular abnormalities, and in one case, directly related to known alteration of AD neurons. Therefore, comparisons of AD and DS iPSCs can now be used to identify underlying genes and pathways that are causative for AD.

2 Methodological Considerations for iPSC Studies

While comparisons between iPSC-derived neurons/glia and DS/AD patient tissue samples may provide the most promising avenue for uncovering mechanisms of disease, iPSC technology is in its infancy and it is necessary to understand the sources of variability we can expect from in vitro studies prior to moving to in vivo/ex vivo exploration. There are multiple factors in iPSC studies that introduce variability and affect the ability to compare data from multiple studies including patient differences, iPSC reprogramming methods, and neuronal differentiation paradigms. Inherent genetic variation among individuals due to genetic diversity as well as disease presentation presents a major challenge to iPSC disease modeling [37, 38]. Further, epigenetic and copy number diversity add another layer of complexity [39] which may plague iPSCs to a greater extent than other samples. Many of these problems can be overcome by using cells from enough different individuals to enable statistically meaningful results. Alternatively, either engineered or spontaneously-generated isogenic cell lines can provide a more practical alternative to limit genetic diversity. For investigations of

single gene mutations, genetic modifications to repair these defects have been sufficient to reverse cellular phenotypes [40]. New genetic technologies such as TALENs and CRISPR/Cas9 will likely play a major role moving forward with in vitro detection of disease phenotypes [41–44]. In addition, new methods allow the silencing of entire chromosomes to correct aneuploidy [45], paving the way for complex gene regulatory analyses.

A potential confounding factor for iPSC research in general, and for comparing data across multiple cells lines from different laboratories, is the method of choice for reprogramming. While most published studies have used integrating retroviruses [30], recent studies have also utilized non-integrating viruses such as Sendai virus [46] as well as episomal vectors [47] to deliver the reprogramming factors Oct4, Klf4, Sox2, cMyc (OKSM). However, while integration of retroviruses has been postulated to cause genomic instability and transcriptional alterations, no evidence of this has been reported to significantly alter neuronal differentiation or identification of disease phenotypes. Additionally, exogenous retroviruses are quickly suppressed (within weeks) in newly-generated iPSCs, leading to activation of the endogenous OKSM factors [30], but silencing does not appear to be required for directed differentiation [48]. Thus, while newer methods are quickly adopted by iPSC researchers, little data exist to suggest retrovirus use is detrimental to the study of iPSCs and their differentiated progeny, and use of "original" cell lines should continue. Additionally, the somatic cell source has been a point of debate for iPSC researchers. While skin fibroblasts as the somatic cell source still dominate in the published literature due to ease of procurement and reliable reprogramming, successful creation of iPSCs has been demonstrated from other cells including lymphocytes [49].

Perhaps the largest determinant of variability for comparison of iPSC studies with each other, and across model systems, is that of differentiation method and resulting neuronal populations. Directed differentiation using exogenous factors has been established to generate numerous transmitter- and region-specific neuronal subtypes including midbrain dopamine (DA), spinal motoneurons (MNs), medium spiny neurons (MSNs), basal forebrain cholinergic neurons (BFCNs), and forebrain (FB) cortical-like glutamatergic/GABAergic neurons. It is generally agreed that for cell replacement therapies, it will be critical to match the transplanted neuronal subtype with that of the primary degenerative phenotype (e.g. DA neurons for Parkinson's disease, MSNs for Huntington's, etc.). This is likely true for mechanistic studies as well. While many other lineages have been generated [50–52], we have outlined the most well-characterized protocols for generation of dorsal and ventral FB (including cholinergic neurons), DA, BFCNs, and MNs in Fig. 1, as these are of primary importance for a number of neurological disorders, including DS and AD.

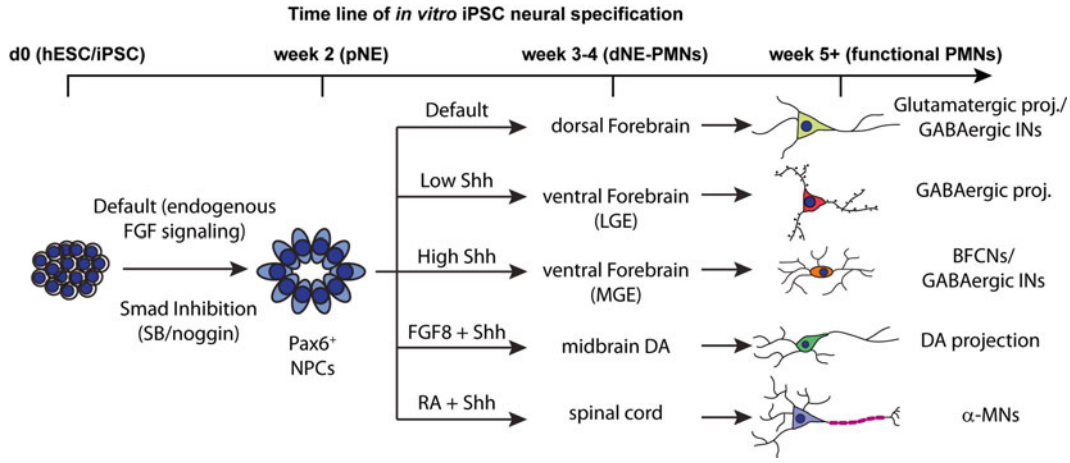

Fig. 1 Directed differentiation of human pluripotent stem cells to transmitter- and region-specific neuronal subtypes. Human pluripotent stem cells (hESC or iPSC) can be differentiated into PAX6+ primitive neuroectoderm (pNE) within 2 weeks in culture either by relying on endogenous FGF signaling or by dual SMAD inhibition. Without additional morphogens (Default), these pNE will differentiate into dorsal forebrain neurons (Glutamatergic and GABAergic). pNE can also be patterned to various lineages via exposure to exogenous patterning factors. Addition of a ventralizing factor such as sonic hedgehog (Shh) allows cells to retain a forebrain phenotype, but will induce ventralization to GABAergic interneurons (INs) and basal forebrain cholinergic neurons (BFCNs). Treatment of pNE with FGF8 and Shh results in differentiation of midbrain dopaminergic (DA) neurons. Treatment of pNE with caudalizing factors such as retinoic acid with Shh can lead to motor neuron (MN) specification

Most recent studies use one of two primary methods to initiate differentiation to an ectodermal lineage. For human embryonic stem cells (hESCs) and iPSCs that are inherently primed toward the neural lineage, exposure to minimally supportive media (e.g. DMEM/F-12 + N2 supplement) is sufficient to allow differentiation along a "default" program that includes a primitive neuroectodermal fate defined by robust expression of the paired homeobox 6 (*PAX6*) gene [53, 54]. This method, pioneered by Su-Chun Zhang at the University of Wisconsin-Madison, has been shown to rely on endogenous FGF acting via FGF receptors to activate the MAPK pathway [55, 56]. A second method, introduced by the Studer Laboratory at Sloan Kettering, demonstrates that hESCs and iPSCs can be directed to ectoderm by inhibition of the Smad pathway using inhibitors of the transforming growth factor beta (TGF-β) and activin/nodal signaling [57]. Both of these methods produce robust *PAX6* expression in early neuroectodermal cells within the first 10 days of differentiation. If no other factors are present during subsequent stages of differentiation the primitive *PAX6*$^+$ neural progenitor cells (NPCs) will proceed through a definitive neuroectodermal stage and go on to become dorsal forebrain (dFB), cortical-like neurons, and then astrocytes with prolonged culturing periods [58]. These dFB neuronal populations typically include robust

numbers of excitatory glutamatergic and inhibitory GABAergic cells (Fig. 1, upper pathway). Interestingly, recent reports suggest that a combination of retinoic acid and dual Smad inhibition can enhance glutamatergic projection neuron differentiation [59]. As retinoic acid is a potent neural inducer, it may enhance early-born neurons to differentiate, mimicking endogenous retinoid signaling from meningeal cells in the developing cortex [60].

It is now generally agreed that *PAX6*$^{+}$ NPCs can also be patterned to various lineages via exposure to exogenous patterning factors [61, 62]. For example, treatment of primitive NPCs with caudalizing factors such as retinoic acid can induce Hox gene expression, which is required for the establishment of spinal cord fates. Additional treatment using a ventralizing factor such as sonic hedgehog (Shh) can lead to the induction genes required for MN specification such as *HB9*, acetylcholine (ACh; the MN neurotransmitter), and *ISL1* (Fig. 1, lower pathway). Similarly, omission of the caudalizing factors during Shh treatment allows cells to retain a FB phenotype, but will induce ventralization to GABAergic interneurons (INs) and BFCNs (Fig. 1, pathways 3–4). This is thought to involve a gradient of Shh treatment both in terms of concentration and timing. Early, high Shh levels will bias the cells toward more ventral fates, leading to increased proportions of BFCNs [63], while moderate Shh treatment allows for increased production of GABAergic interneurons that derive from the medial and lateral ganglionic eminences [64].

It is critical to note that gene expression patterns in cells of various regional and transmitter phenotypes differ substantially from one another, as indicated by changes in differential expression of unique markers. This underscores the fact that these are truly distinct populations, highlighting the need to compare similar populations between control and disease conditions, as well as across studies, to reveal relevant disease phenotypes. While AD affects many neuronal populations as disease progression enters later stages, neocortical and cholinergic neurons are the primary affected population during early phases in AD patients and animal models [65]. Thus, to understand how early deficits in DS neurons may underlie later problems in AD it is important to generate appropriate neuronal populations, such as cortical-like neurons.

3 Defining Mechanisms Underlying Early Alzheimer's Disease-Like Pathologies in Down's Syndrome

3.1 Pathology and Gene Expression of AD iPSC-Derived Neurons

Three recently published studies examined hallmarks of AD pathology as well as transcriptome analysis [32–34] using iPSC-derived neurons generated from fibroblasts taken from AD patients, as well as non-demented controls (NDCs). Interestingly, these reports took advantage of very different patient populations of familial AD

in an effort to reveal molecular deficits in AD neurons. The first report from the Suzuki laboratory created AD iPSCs from patients with autosomal-dominant mutations of the presenilin genes [34] while Inoue and colleagues [33] chose patients who expressed autosomal-recessive mutations of the *APP* gene. In contrast, Goldstein and colleagues created AD iPSCs from patients overexpressing *APP* (APP^{Dp}), which may have the most relevance to studies of DS [118]. In addition, two reports generated iPSCs from sporadic AD patients, although gene expression was not assessed from these samples [32, 33]. Because this chapter is focused on gene expression changes across relevant cellular populations affected in both DS and AD (neurons), we will target our discussion to those studies that performed global transcriptome analyses related to the familial cases as they relate to general AD phenotypes and how gene expression from DS studies may help inform AD pathophysiology.

As mentioned, the magnitude of gene expression changes between two iPSC-derived cell populations will primarily reflect the differentiation state of the cells, while disease phenotypes can be expected to be smaller in magnitude. Goldstein and colleagues [118] found that neurons from APP^{Dp} and sporadic AD patient-derived iPSCs showed significantly elevated Aβ, p-Tau, and GSK-3β, a key kinase involved with post-translational modifications of the amyloid and Tau proteins, as well as defects in the formation of early endosomes, all hallmarks of AD patients and animal models [66, 67]. However, reported gene expression patterns from neurons that are directly relevant to AD are likely to be subtle due to the choice of differentiation paradigm. Israel and colleagues co-cultured neural progenitor cells with PA6 cells, a method that generates a large proportion of midbrain DA neurons (Fig. 1; [68, 69]). In addition, their data are compared with fetal brain samples that are not described in detail but likely represent a mixed population with a majority of cells from the cerebral cortex. Examination of the gene expression data reveal significant expression of midbrain and DA neuronal markers such as Iroquois homeobox factors [70] and tyrosine hydroxylase in the iPSC-derived neurons compared with brain tissue. In contrast, fetal brain samples showed enriched expression of cortical markers such as *SATB2*, *LHX2*, *TBR1*, and *EMX2* [32]. It is not surprising then, that the neuronal population analyzed contains relatively minimal proportions of glutamatergic/GABAergic neurons as measured by immunocytochemistry and physiology [32]. Thus, it is difficult to assess the changes that are specific to AD cells rather than those that are due to neuronal specification when comparing this dataset and other DS/AD cortical neuronal populations (see below). However, as the iPSCs and their neuronal derivatives carried a duplication of the *APP* locus (APP^{Dp}), APP transcript levels were significantly upregulated compared with NDCs and fetal brain samples that carried no know genetic abnormalities.

In contrast to the methods used by Israel and colleagues, Kondo et al. [33] use differentiation methods similar to those developed by the Studer laboratory [57], using inhibitors of TGF-β and activin/nodal signaling to initiate differentiation and generate cortical-like cells [71]. In their study, patient fibroblasts contained two different mutations of the APP gene (APP-E693Δ and APP-V717L), but possessed only two copies. Therefore, no significant increase in APP transcript was observed in their AD iPSC samples. However, both mutations demonstrated robust, neuron-specific effects on Aβ levels and the production of reactive oxygen species, which were blocked by inhibitors of GSK3β. Kondo et al. [33] also performed transcriptome analysis on samples of neurons carrying the APP-E693Δ, a rare, autosomal-recessive mutation that causes early-onset AD but without extracellular Aβ plaque deposition [72]. Interestingly, only 50 identified genetic loci were differentially regulated (>1.5-fold) in AD neurons, some previously implicated in AD but many novel transcripts as well. Significant increases were observed in oxidative stress (OS) genes such as peroxiredoxin, oxidoreductase and peroxidase activities for neurons carrying the APP-E693Δ mutation, pathways that have previously been implicated in AD [11]. In contrast, a number of β-glucuronidase isoforms are down-regulated, suggesting that multiple metabolic pathways are disrupted that may involve mitochondrial, ER, and golgi functions. In addition, Kondo et al. [33] observed a number of synaptic/cell adhesion markers, zinc finger proteins, and regulators of apoptosis were altered as well. Thus, these AD iPSC studies identified multiple deficits that are hallmarks of AD in neurons as well as novel changes, supporting the use of the iPSC platform even for a disease that takes decades to manifest in human patients.

3.2 DS iPSCs as a Model of Early AD

As mentioned, DS patients represent a unique population of individuals that may help to uncover early deficits of AD pathology. To date, three reports have explored various aspects of DS from iPSC-derived neurons [73–75], and each has focused on cortical-like neurons, with subtle differences in methodology. For instance, while Weick and colleagues used "default" methods to generate mixed populations of excitatory and inhibitory neurons [75], Shi and colleagues used methods to enrich for excitatory glutamatergic neurons [74], while Briggs and colleagues used the dual SMAD inhibition method [73]. All three studies used different methods of reprogramming, from retroviral and sendai virus transduction, as well as episomal methods. Interestingly, despite evidence for aberrant neuronal differentiation in DS brain [76–84], none found that DS iPSCs were deficient in their ability to differentiate to neuroepithelia and post-mitotic neurons compared with control cells. Thus, the results from these studies are can be directly compared, with a relatively high degree of confidence that methodological differences play a minimal role in the differences observed (see below).

DS iPSCs and their neuronal derivatives display aberrant phenotypes consistent with previous human and animal studies of DS neurodevelopment as well as phenotypes consistent with early AD pathology [73–75]. All studies found increased expression of APP in DS neurons, which would suggest that increased APP is available for proteolytic processing in these cells [73–75]. In fact, Shi et al. [74] found that DS neurons from both iPSCs and hESCs demonstrated elevated Aβ species (both 40 and 42), a decreased Aβ40:Aβ42 ratio, amyloid aggregate formation, as well as hyperphosphorylated tau in neurons cultured for extended periods (>60 days). Therefore, DS iPSC-neurons display similar phenotypes to AD iPSC-neurons. Similarly, all studies found that DS iPSCs and neurons exhibited significant increases in OS markers and/or increased sensitivity to reactive oxygen species (ROS) challenge. Metabolism and oxidative stress have been consistently reported as an underlying target of dysfunction in both DS and AD [11, 85].

Loss of synapses represents a major clinical feature of AD progression and synaptic abnormalities are correlated with both human and animal models of DS [86–89]. As for human DS iPSC-derived neurons, it was shown that both excitatory and inhibitory synaptic activity was diminished in DS neurons compared with controls [75]. This was paralleled by a decrease in synapsin-1 punctae on DS neurites, indicating either a failure to form similar numbers of synapses, or instability of synaptic junctions, which are then subsequently lost. In contrast, no deficits in synaptic punctae were found by Shi et al. [74], who analyzed the proportion of synaptophysin and PSD-95 doubled labeled punctae along iPSC-derived neurites. While Israel et al. performed some quantification on the physiological properties of AD neurons they also did not find differences between control and AD cells and concluded that "extended culture periods may be required to study Alzheimer's disease-associated loss of synaptic proteins" [118]. Because all investigations examined relatively early timepoints (<100 days in vitro), more functional data is needed on the iPSC-derived neurons from individuals with AD and DS to determine whether this is a repeatable phenotype in cultured cells.

3.3 Gene Expression Changes in DS Related to AD

Due to trisomy of Hsa21 in DS, in terms of number of genes altered, transcriptome changes have been found to be primarily a function of gene duplication of the genes on Hsa21 [90]. However, the largest magnitude of transcript changes primarily occur on genes located on autosomes and sex chromosomes other than Hsa21 [90–93]. Previous Gene Ontology (GO) analyses have pointed to alterations in gene families involved with the usual suspects (APP, Aβ and Tau), as well as oxidative stress, neuronal differentiation, a variety of second messenger cascades, and synaptic development/loss [91, 94, 95].

These findings are largely recapitulated in DS iPSCs. Interestingly, both Briggs et al. [73] and Weick et al. [75] developed isogenic control lines that were disomic for Hsa21. This fact allowed expression arrays of DS cells with reduced background "noise" due to genetic variability. Both studies found significant gene dosage effects of Hsa21 genes, with similar numbers of genes overexpressed (63 and 125, respectively), with a small number underrepresented (7 and 14, respectively). The differences in number primarily reflect the cutoff value of the analysis. Increasing the cutoff of the Weick et al. dataset [75] from 1.2 to 1.5-fold reduces the number of overexpressed genes to 60, nearly identical to the number reported in Briggs et al. [73]. Interestingly, greater than 60 % of the altered genes were identical in the two datasets. However, some interesting differences are noteworthy. While APP and *DYRK1A* were both upregulated in iPSCs in the study by Weick et al. [75], APP was not changed and *DYRK1A* was significantly *downregulated* in Briggs et al. [73]. Furthermore, 40 % of upregulated genes differed between the populations and none of the downregulated genes were shared between the datasets, suggesting significant differences in genetic regulation between iPSC lines with the same underlying genetic defect.

With regard to common expression changes in DS and AD neuronal populations, we will focus our discussion by comparing data from Weick et al. [75] with the data obtained from Kondo et al. [33] as these two studies produced neurons of similar phenotypes (i.e. forebrain). To more accurately assess the utility of DS iPSC-derived neurons to inform early AD pathology we directly compared the expression arrays from both datasets. The microarray gene expression data sets from the two studies were generated using different Affymetrix platforms. Weick et al. [75] using GeneChip Human Genome U133 plus 2.0 Array while Kondo et al. [33] used GeneChip the Human Gene 1.0 ST Array. The major difference between the two platforms lies in the fact that former interrogates a few hundred bases proximal to the 3′ end of each mRNA species to approximate expression of the entire gene whereas the latter queries the entire transcript of each gene. Despite this difference, gene expression measured in both platforms are highly concordant [96] which makes it possible to compare the results from both platforms and also integrate the data sets into one analysis.

In this analysis we considered a number of given Gene Ontology (GO) terms such as response to reactive oxygen species (GO:0000302) and cellular response to oxidative stress (GO:0034599), processes known to be affected in both DS and AD. We tried to identify genes annotated at each of these GO terms that are differentially expressed between diseased samples and controls in both data sets. The analysis can be divided into low-level analysis (data preprocessing) and high-level analysis (statistical differential expression analysis).

Affymetrix arrays (in both platforms) use multiple probes to measure the same transcript. One low-level analysis step is to summarize these repeated measurements into a single value for each probeset while removing undesired sources of variation so that the resulting single values (estimates for gene expression level) of all the probesets reflect the true changes in mRNA abundance as accurately as possible. This was achieved in our analysis through the use of the robust multi-array average (RMA) [97] algorithm implemented in Bioconductor package oligo. The RMA was performed on the two data sets separately because of the difference in the platform.

The second step of the data preprocessing is to match the probesets between the two data sets [98]. Affymetrix provides a matching file available on the company's website which has 29,129 mappings corresponding to unique U133 plus 2 probeset IDs; for each of the 29,129 probesets in the DS data set we could find one and only one corresponding probeset in AD data set. Each pair of the matched probesets represents the same gene. We restricted our analysis to these 29,129 probesets of the first data set and corresponding probesets in the second data set and called them as matched data sets. We next performed the high-level analysis (i.e. differential expression analysis) to identify the genes that are differentially expressed in both data sets.

For each of the given GO terms we obtained all Affymetrix probesets that are annotated at that node, either directly or by inheritance, using function 'lookUp' of the Bioconductor package 'annotate'. The differential expression analyses were performed on the overlap of these probesets and those available in the matched data sets. In order to identify the genes that are differentially expressed in both data sets, we first performed student t-test for each probeset on both data sets, separately. Then we rank-ordered the maximum of the two p-values and considered the genes with smallest maximum p-values as significant if $p < 0.1$. This value was chosen due to the stringency of the comparisons in this high-level analysis and small number of samples in each group.

The data is illustrated by Gene Ontology (GO) results in Table 1, which indicates significant overlap in the pathways that are disrupted in both cell types. We performed GO analysis on terms with previously-indicated relevance to each disorder, and noticed significant overlap in the number of genes shared between the two datasets (column 5). For instance, for all GO terms examined, the number of genes shared between DS and AD (when present) had an average of 22.6 % overlap. Overall, 115 genes were found to be significantly altered in both datasets. Notable genes include *CAT*, *ITSN1*, *MAP2*, *MAPK1*, *PRKAR2A*, *PSEN2*, *RAB4A*, and *STX7*. These genes are involved with cell cycle regulation, oxidative stress, synaptic transmission, endosomal trafficking [99], and signal transduction from plasma membrane to the nucleus. Remarkably, significant similarities were found between the two datasets despite

Table 1
Comparison of microarray data from DS iPSC-derived neurons with AD iPSC-derived neurons

GO pathway	Total genes	Matched (analyzed)	Changed (AD)	Changed (DS)	Overlap (same genes)
Synaptic transmission	1,529	1,143	231	90	22
MAPK cascade	1,373	987	164	100	15
Glycosylation	591	413	66	45	6
Aging	506	379	79	34	6
Neuron apoptotic process	436	335	60	38	5
Oxidative stress	296	205	41	15	6
ATP metabolic processes	261	186	23	20	2
Synapse Assembly	188	142	27	15	3
Mitochondrial permeability	99	80	10	8	0
Beta-amyloid metabolism	36	25	4	2	1

Direct comparison of the expression arrays from Weick et al. [75] with the data obtained from Kondo et al. [33]. Gene Ontology (GO) terms of processes known to be affected in both DS and AD were analyzed in the datasets. Table shows a significant overlap in the pathways that are disrupted in both cell types

the lack of APP duplication in the AD iPSC-derived neurons. This result points to common pathways engaged by very different underlying mechanisms of AD pathogenesis.

Dysfunction of endosomes has been proposed to represent one of the earliest shared phenotypes of DS and AD, when Aβ levels are relatively low [66]. The retrograde signaling of neurotrophins through endosomal trafficking, specifically nerve growth factor (NGF), has been implicated in the neuronal cell death in AD and DS [100–103]. Proper NGF signaling requires endocytosis and retrograde transport, which is associated with activated components of the Ras-MAPK pathway located on endosomes. Additionally, members of the Rab family of GTPases play an integral role in the local processing of proteins during synaptic vesicle release and recycling. The syntaxins are a family of proteins involved with diverse vesicular docking and fusion events between various targets including the plasma membrane and other intracellular compartments. Interestingly, both *STX7* and *ITSN1* have been specifically associated with endosomal and lysosomal compartments [104–106], and *ITSN1* is known to interact with several proteins involved with synaptic vesicle recycling [107–109]. Thus, the simultaneous dysregulation of *MAPK*, *ITSN1*, *PSEN2*, *RAB4A*, and *STX7* supports the idea that endosomal signaling may be perturbed in both DS and AD neurons at early timepoints.

The presence of altered *PSEN2* is particularly interesting not only because it is expressed in intracellular vesicles, but because mutations in the Presenilin genes (*PSEN1* and 2) are strongly associated with familial AD [110] Multiple mutations in *PSEN1* and *PSEN2* are known to cause early-onset AD between the ages of 60–65 [111]. *PSEN1* and PSEN2 are part of the γ-secretase complex, which cleaves a number of membrane proteins, including APP. This proteolytic cleavage of the C-terminal end of APP, along with a second, N-terminal cut is required for production of the Aβ peptide [119]. While normal β-secretase activity primarily leads to the Aβ40 form, a small amount of Aβ42 can also be produced, which is more prone to aggregation and can cause neuronal damage. Mutations in *PSEN* lead to significant overproduction of the Aβ42, and an increase in the Aβ42/Aβ40 ratio, resulting in AD. Thus, it is curious to observe overproduction of a presumably normal *PSEN2* in these populations of DS and AD neurons. In the AD cells, a clear increase was observed in the Aβ42/Aβ40 ratio [33], while this was not studied in Weick et al. [75]. However, the DS neurons in Shi et al. [74] demonstrated significant increases in both Aβ species and alterations in the Aβ42/Aβ40 ratio. Thus, it is likely that overall increased processing of APP cleavage by *PSEN2*/β-secretase can lead to toxic levels of Aβ42.

In addition, a number of transcriptional regulators were found to be altered in both datasets, including *SP3*, *TLX2*, and multiple zinc finger proteins (*ZNF*s *22*, *248*, *37A*, *439*, *510*, and *675*). Interestingly, both *SP3* and *TLX2* have both been previously implicated in AD pathology. Using Baysian network analysis of six different datasets consisting of a total of 110 patients (62 AD and 48 controls), Yoo and Yoo identified altered expression of four genes, including *TLX2*, that showed the highest association with disease incidence [112]. Further, a study by Boutillier and colleagues showed that both *SP3* and *SP4* were both upregulated at the protein level, and associated with NFTs, in postmortem AD brains [113]. The ZNFs identified are part of a family that represents one of the most abundant proteins in eukaryotic genomes and have incredibly diverse functions [114]. However, many require the binding of zinc (Zn^{2+}) or other metals in their finger-like protrusions to regulate DNA transcription of a host of genes. The fact that most of the transcription factors identified here belong to the ZNF family, including *SP3*, suggest a general pattern of altered transcriptional response which is correlated with altered metal metabolism. While the metal hypotheses of AD suggest that direct interactions of copper (Cu^{2+}) and zinc (Zn^{2+}) with extracellular Aβ increase aggregation at the synapse [115], it is possible that changes in the availability of metal ions result from alterations in transcription factors that require these ions for activity within the nucleus.

Lastly, it is noteworthy to point out the absence of both *DYRK1A* and APP in this dataset. While there is an approximately

1.5-fold increase in APP in DS samples [75], the APP-E693Δ mutation does not lead to increased transcript expression. Thus, the lack of significance for APP expression change is not surprising when looking at the intersection of the two different data sets. However, the absence of *DYRK1A* is not as easily explained. As mentioned the *DYRK1A* gene is located on Hsa21, and is a serine/threonine protein kinase capable of phosphorylating tau protein at 11 serine and threonine residues, as well as threonine 212, a site that may prime it for further phosphorylation events by GSK-3β [28]. Moreover, DYRK1A protein levels have been found to be overexpressed in multiple AD patient tissue samples [116]. It has been hypothesized that inhibition of DYRK1A may be a potential treatment of the developmental defects of DS as well as the progression of AD pathology. Due to the lack of *DYRK1A* expression alterations in AD iPSC-derived neuronal samples may indicate that while it is important for DS-related AD pathologies it is not an early marker for all AD patients.

4 Future Perspectives

While the current iPSC studies provide proof of concept that both DS and AD can be modeled in a dish, additional gene expression analyses are needed to uncover underlying genes and pathways that are causative for AD but that are not confounded by the issues of sample variability stated previously. For example, it will be interesting to examine gene expression data from cortical-like neurons derived from familial AD patients that carry the APP^{Dp} duplication [32]. This single gene duplication is sufficient to induce early-onset AD symptoms in patients as well as both NFT and amyloidogenic pathology in iPSC-derived neurons. Thus, transcriptome analyses between these and DS neurons should provide an excellent platform to increase the signal-to-noise ratio for early expression changes that play causative role in the development of dementia. Coupling cross-comparisons of multiple datasets like the one performed here, along with analyses of the APP-V717L mutation [33], should strengthen identification of genes with an obligatory role rather than those that may be secondary to disease onset.

Whole-genome sequencing and large-scale genome-wide association studies of large populations will also assist in uncovering any underlying single nucleotide polymorphisms and gene mutations that link DS and AD at a single gene level. It may be that these types of studies, will both identify shared features of AD-like pathology as well as accelerate the segregation of various types of AD into categories based on molecular dysfunction.

As recently as 20 years ago, the life expectancy for individuals with DS was only 25 years. Since then, the life expectancy has risen to greater than 50, due in large part to the reduced institutionalization of individuals with DS and greater awareness and care for

individuals with developmental disorders in our society [16]. With the increased lifespan come additional health issues for DS individuals including premature aging and the development of AD. Yet, this situation also provides a potential resource for learning more about the development of AD. The recent implementation of the Down Syndrome Consortium Registry (DS-Connect) by the U.S. National Institutes of Health will enable researchers access to detailed information about DS individuals and provide research subjects that are likely in early stages of AD [117]. It is possible that DS may represent a single underlying cause of AD pathology that will only relate to a minority of AD patients. Nonetheless, information gleaned from studying DS will undoubtedly provide insight into early manifestations of AD neuropathology.

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